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DIASTOLIC, SYSTOLIC AND SARCOPLASMIC RETICULUM [Ca^{2+}] DURING INOTROPIC INTERVENTIONS IN ISOLATED RAT MYOCYTES

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SUMMARY

1. The fluorescent indicator Fura-2 has been used to monitor intracellular [Ca^{2+}] (Ca_i^{2+}) in myocytes isolated from the ventricles of rat hearts.

2. The relationships between diastolic Ca_i^{2+}, systolic Ca_i^{2+} and the Ca^{2+} content of the sarcoplasmic reticulum (SR; assayed using caffeine) have been studied during changes of stimulation rate and bathing [Ca^{2+}] (Ca_o^{2+}).

3. When stimulation rate was increased, there were increases in diastolic Ca_i^{2+}, systolic Ca_i^{2+} and the Ca^{2+} content of the SR.

4. The SR inhibitor ryanodine (1 μmol l^{-1}) decreased the size of the Ca_i^{2+} transient, and abolished the increase of Ca_i^{2+} produced by caffeine (10 mmol l^{-1}). In the presence of ryanodine, increasing stimulation rate increased diastolic Ca_i^{2+} but not systolic Ca_i^{2+}.

5. Increasing Ca_o^{2+} led to increases of diastolic Ca_i^{2+}, systolic Ca_i^{2+} and SR Ca^{2+} content similar to those observed during changes in stimulation rate.

6. Ryanodine altered the relationship between systolic and diastolic Ca_i^{2+} during changes of Ca_o^{2+}.

7. These results are consistent with a change of diastolic Ca_i^{2+} leading to an increase in the Ca^{2+} content of the SR, and hence an increase in the size of the Ca_i^{2+} transient during changes in stimulation rate and Ca_o^{2+}.

INTRODUCTION

Contraction of cardiac muscle is initiated by a transient rise in cytoplasmic [Ca^{2+}] (Ca_i^{2+}). This Ca^{2+} appears to come from the extracellular space (via the Ca^{2+} current (I_{Ca}) and the Na^+–Ca^{2+} exchange mechanism) and the sarcoplasmic reticulum (SR), although the contribution made by each of these to the Ca_i^{2+} transient probably varies between species (Bers, 1985). The magnitude of this rise in Ca_i^{2+}, and hence the strength of contraction, can be altered by inotropic interventions, such as changes in stimulation rate, bathing [Ca^{2+}] (Ca_o^{2+}) and the application of drugs (e.g. Allen & Kurihara, 1980). What are less clear are the mechanisms that underlie changes in the size of the Ca_i^{2+} transient, and hence the strength of contraction, during such interventions. The possibilities are: first, that changes in the amount of Ca^{2+} entering across the cell membrane may alter the size of the Ca_i^{2+} transient, and hence the

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strength of contraction, either by activating the myofilaments directly (Bers, 1985) or by triggering the release of a different amount of Ca^{2+} from the SR (Fabiato, 1985); second, that the amount of Ca^{2+} available for release from the SR changes, either because of a change in the time since the previous Ca^{2+} release (i.e. the time allowed for restitution of the Ca^{2+} release process; Braveny & Kruta, 1958) or because the SR Ca^{2+} content has changed (i.e. a change in the Ca^{2+} loading of the cardiac cell; e.g. Smith, Valdeomillos, Eisner & Allen, 1988).

It has, for example, been suggested that a number of interventions (e.g. increasing stimulation rate) lead to Ca^{2+} loading by elevating intracellular $[Na^+]$ (Langer, 1968; Eisner, Lederer & Vaughan-Jones, 1981; Boyett, Hart, Levi & Roberts, 1987) and hence, via the Na^+–Ca^{2+} exchange mechanism, increasing cytoplasmic (Lado, Sheu & Fozzard, 1982; Sheu & Fozzard, 1982; Harding, Kirschenlohr, Metcalfe, Morris & Smith, 1989), and hence SR $[Ca^{2+}]$ (Smith *et al.* 1988), and it is this that leads to an increase in the size of the Ca_i^{2+} transient during these interventions. However, the relationships between diastolic Ca_i^{2+} and the size of the Ca_i^{2+} transient and SR Ca^{2+} content remain unclear. Previous studies that have monitored diastolic and systolic Ca_i^{2+} (e.g. Lee & Clusin, 1987; Lee, Smith, Mohabir & Clusin, 1987) have used multicellular preparations in which it is difficult to correlate systolic and diastolic Ca_i^{2+} accurately, since Ca_i^{2+} is monitored from several cells whose individual contributions are not known. The relationship between cytoplasmic Ca_i^{2+} and SR $[Ca^{2+}]$ is also unclear. The amount of Ca^{2+} released from the SR of ferret papillary muscles by caffeine shows changes that are qualitatively similar to the observed changes of systolic Ca_i^{2+} during different inotropic interventions (Smith *et al.* 1988), but the increase of Ca_i^{2+} produced by caffeine is markedly smaller than that produced by the action potential. In contrast, in rat trabeculae the amount of Ca^{2+} released from the SR by rapid cooling appears to remain constant during changes in the size of the contraction (Bouchard & Bose, 1989).

The present study was undertaken, therefore, to investigate the relationships between systolic Ca_i^{2+}, diastolic Ca_i^{2+}, and SR Ca^{2+} content in heart muscle by using the fluorescent indicator Fura-2 (Grynkiewicz, Poenie & Tsien, 1985) to monitor Ca_i^{2+} in single myocytes isolated from the ventricles of rat hearts. Caffeine, which releases Ca^{2+} from the SR (Weber & Herz, 1968), has been used to assay SR Ca^{2+} content.

The results show that during changes in stimulation rate and Ca_o^{2+} there are parallel changes in diastolic, systolic and SR $[Ca^{2+}]$. In the presence of the SR inhibitor ryanodine, the changes in diastolic Ca_i^{2+} still occur but the changes in systolic Ca_i^{2+} and SR Ca^{2+} content were much reduced. These data are consistent with a change in Ca_i^{2+} leading to an increased SR Ca^{2+} load, and hence an increase in the size of the Ca_i^{2+} transient.

Preliminary reports of some of the present observations have already appeared (Frampton, Orchard & Boyett, 1990*a*, *b*).

METHODS

Isolation of ventricular myocytes and loading with Fura-2

Adult rats (250–300 g) were deeply anaesthetized with chloroform. The animal was then exanguinated and the heart removed and transferred to a beaker containing a HEPES-buffered physiological salt solution (PSS; see below for composition) and heparin (1 ml (100 ml PSS)$^{-1}$),

where it was gently massaged to remove excess blood. The heart was then Langendorff-perfused at constant flow with PSS containing Ca^{2+} (0·75 mmol l^{-1}) at 37 °C. The flow rate was set at 8 ml min^{-1} (g wet weight)$^{-1}$ (assuming heart weight to be 0·5% of body weight).

The perfusion pressure was monitored continuously, and was typically 30–40 mmHg. Once the preparation appeared stable, perfusion was switched to a nominally Ca^{2+}-free PSS for 5 min. During this time, the heart stopped contracting and there was a small but steady increase in perfusion pressure (cf. Levi, Price, Hall & Kaufman, 1990). The heart was then perfused with PSS containing collagenase (1 mg ml^{-1}; Worthington, type II), protease (0·1 mg ml^{-1}; Sigma, type XIV) and Ca^{2+} (50–100 μmol l^{-1}). This solution was recirculated to give a total exposure to enzyme of 8·5–9 min. During the enzyme perfusion, there was a marked increase in perfusion pressure (typically up to 90–100 mmHg) which then slowly returned towards the initial level (30–40 mmHg).

At the end of the enzyme perfusion, the heart was cut down and the ventricles dissected free. The ventricular walls were cut from the atrioventricular junction to the apex and splayed out. Meanwhile, the enzyme-containing PSS was collected and to it was added sufficient bovine serum albumin to make a 2% solution. This solution was then shaken with the cut ventricular tissue for 5 min at 37 °C. This mixture was filtered through gauze and the filtrate centrifuged at 400 r.p.m. for 30 s. The supernatant was removed and the cell pellet was resuspended in PSS containing Ca^{2+} (0·5 mmol l^{-1}) and allowed to settle again at room temperature. This process was repeated until all the ventricular tissue was fully digested.

The ventricular myocytes were not exposed to 1 mmol l^{-1} Ca_o^{2+} until at least an hour after the isolation procedure and following loading with Fura-2 AM, the acetoxymethyl ester of Fura-2. Myocytes were incubated in PSS containing Ca^{2+} (0·5 mmol l^{-1}) and Fura-2 AM (5 μmol l^{-1}) for 12–15 min at room temperature. The cells were then centrifuged, the supernatant removed, and the cells resuspended in PSS (containing 1 mmol l^{-1} Ca^{2+}) and kept at room temperature until they were used (normally less than 4 h after loading).

All the cells chosen for study showed clear striations, were normally quiescent and responded to stimulation with a rapid twitch.

Apparatus

A schematic diagram of the optical system for the measurement of both cell shortening and Fura-2 fluorescence is shown in Fig. 1. Ventricular myocytes were allowed to settle on the glass coverslip bottom of a superfusion chamber mounted on the stage of a Nikon Diaphot inverted microscope, enclosed within a darkened Faraday cage. Solutions were pumped to the chamber at approximately 3 ml min^{-1}. Two input lines were controlled by electrically operated solenoid valves, which enabled a rapid solution change-over (within 4 s). The solution level and drainage from the bath were controlled by an electronic feedback system (Cannell & Lederer, 1986). Experiments were carried out at room temperature (24–27 °C). The cells were stimulated by means of two platinum field electrodes on either side of the bath. The stimulus voltages were typically 40–60 V and of 2 ms duration.

Fura-2-loaded myocytes were alternately excited with ultra violet (UV) light (shown as the thin continuous lines in Fig. 1) of 340 and 380 nm wavelengths. UV light from a 150 W xenon arc lamp (Ealing Electro-optics) was focused by a pair of quartz lenses onto a rotating filter wheel (Cairn). Before the filter wheel, the light passed through a 50% neutral density filter to reduce overall UV light intensity and a heat absorbing filter (Schott UG 5 filter) to protect the excitation filters in the wheel from excessive heat. In addition, a protective shutter (Uniblitz) could be used to block off UV light when it was not required. The wheel housed six bandpass filters – three 340 nm and three 380 nm filters arranged alternately, each with bandwidths of 10 nm – and was normally spun at 100 Hz. Excitation light from the filters was transmitted to the microscope by a flexible liquid light guide (Cuel), which minimized the transmission of mechanical vibration from the filter wheel assembly to the microscope. A 430 nm dichroic mirror beneath the microscope nosepiece reflected the excitation light to the cell under study via a 40× oil immersion FLUOR objective lens (Nikon; numerical aperture 1·3). The resulting Fura-2 fluorescence (shown as the broad continuous line in Fig. 1) was also collected by the objective lens and transmitted to the side port of the microscope where it passed through a variable rectangular diaphragm (Nikon). The diaphragm was arranged so that it only outlined the cell under study, thus ensuring fluorescence from neighbouring cells was not measured. The fluorescence was reflected by a 580 nm dichroic mirror (see below) to

a photomultiplier tube (Thorn EMI 9844B) via a 510 nm emission filter (bandwidth 20 nm), which ensured only fluorescence at about 510 nm was detected. The output of the photomultiplier tube passed to the Cairn spectrophotometer control box which, in addition to controlling the filter wheel speed, correlated the fluorescence signal with the particular excitation filter in the lightpath. The

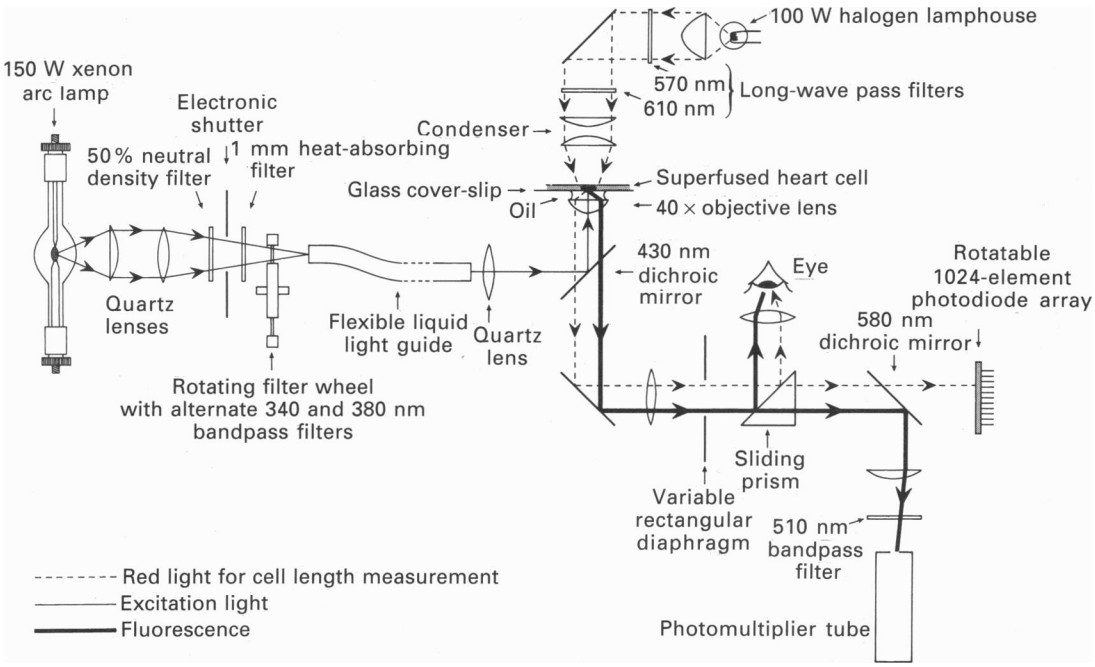

Fig. 1. A schematic diagram of the epi-fluorescence optical system used in the present study (not to scale). For details see text.

three fluorescence signals in response to excitation light from the three 340 nm excitation filters were averaged (340 signal), as were the signals in response to the three 380 nm filters (380 signal). The ratio (340 signal/380 signal, a function of $[Ca^{2+}]$) was determined using a custom-built analog divider circuit and was displayed, with the 340 nm and 380 nm signals, on a chart recorder (Gould) and stored on magnetic tape (Racal 7DS FM recorder) for later off-line analysis. Fluorescence signals were filtered by low pass filters with a cut-off frequency of 100 Hz unless otherwise stated.

To measure twitch contractions, cells were illuminated with long wavelength red light (shown by the dashed lines in Fig. 1) from a 100 W halogen lamp (Nikon). Two long-wave pass filters prevented wavelengths shorter than 610 nm from passing to the cell under study. Thus the red light did not interfere with the measurement of Fura-2 fluorescence from the myocyte. The red light from the cell created an image of the cell which was collected by the objective lens and directed to the side port of the microscope, where it was separated from the Fura-2 fluorescence by the 580 nm dichroic mirror. The cell image was then focused onto a linear 1024-element photodiode array (Reticon). From the output of the array, the length of the cell was measured electronically as described in detail by Boyett, Moore, Jewell, Montgomery, Kirby & Orchard (1988).

Records of Fura-2 fluorescence and cell length were normally averaged and analysed using a Tandon computer fitted with a Data Translation DT2805 A/D board and running 'Vacuum' data acquisition software, sampling each channel at 1 kHz. To obtain continuous records of diastolic and systolic Ca_i^{2+} (Fig. 6), a Tandon computer and a CED (Cambridge Electronic Design) interface running software written in TURBO PASCAL were used.

Estimation of Ca_i^{2+} using Fura-2

There are a number of problems in the calibration of Fura-2 added as the AM ester to cardiac myocytes. First, the Ca^{2+} sensitivity of Fura-2 *in vivo* may be different from that of the free acid *in vitro* (e.g. Scanlon, Williams & Fay, 1987). This probably arises because of the presence of

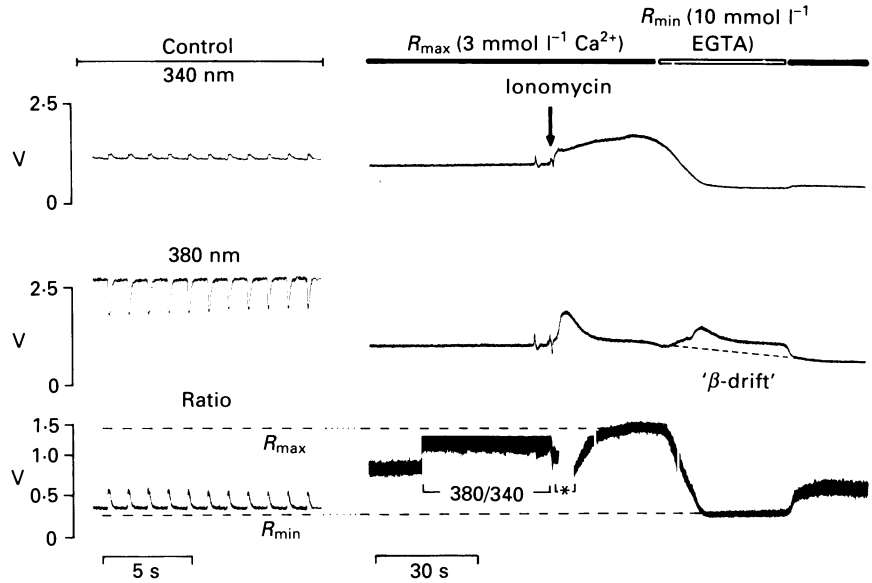

Fig. 2. Calibration of Fura-2 fluorescence. The left panel shows fluorescence changes recorded from a myocyte (stimulation rate 1 Hz) at 510 nm while illuminating the cell alternately with 340 nm (top) and 380 nm (middle) light. The bottom trace shows the on-line fluorescence ratio (which is shown as a 340/380 ratio except where indicated). The right panel shows the fluorescence changes during excitation at 340 nm (top) and 380 nm (middle) during the calibration procedure in the same myocyte. Initially, the cell was metabolically depleted using a solution containing 5 μmol l^{-1} CCCP and 5 μmol l^{-1} rotenone (not shown). Following the cessation of cell contraction there was a marked increase in the fluorescence ratio. The cell was then superfused with 3 mmol l^{-1} Ca^{2+} solution which resulted in a further increase in the ratio. Ionomycin (25 μmol l^{-1}) was added directly to the superfusate. Following an addition artifact (*), there was a marked increase in the fluorescence ratio. The peak of this fluorescence change was taken to represent full saturation of the intracellular Fura-2, i.e. R_{max}. The superfusate was then changed to one containing 0 Ca^{2+} and 10 mmol l^{-1} EGTA. This produced a rapid fall of the ratio to a value which was taken as the value of R_{min}. The use of ionomycin caused Fura-2 to leak from the cell and hence there was a decline in the absolute fluorescence level. Thus, β, the ratio of maximum fluorescence during excitation at 380 nm (recorded at R_{min}) to minimum fluorescence during excitation at 380 nm (recorded at R_{max}) was calculated using an extrapolated value of fluorescence at 380 nm at R_{max} which could thus be directly compared with the measurement of fluorescence at 380 nm at R_{min} (as indicated by the dashed line, 'β-drift').

fluorescent, but Ca^{2+}-insensitive, hydrolysis products of Fura-2 AM (Scanlon *et al.* 1987) and the viscosity of the intracellular compartment (Roe, LeMasters & Herman, 1990). It is necessary, therefore, to establish Fura-2 fluorescence in saturating $[Ca^{2+}]$ and zero $[Ca^{2+}]$ *in vivo*. We have used a calibration technique based on that of Li, Altschuld & Stokes (1987). The protocol is shown in Fig. 2. Briefly, the cell being studied was metabolically inhibited (see below) so that Ca^{2+} homeostatic mechanisms, which may prevent Ca^{2+} equilibration across the cell membrane, would be inhibited. The Ca^{2+} ionophore ionomycin was then added at 25–50 μmol l^{-1} to the superfusate

to equilibrate transmembrane [Ca^{2+}] in the presence of saturating Ca_o^{2+}. When the fluorescence ratio was stable, the superfusate was switched to one containing 10 mmol l^{-1} EGTA (zero [Ca^{2+}]). Thus the fluorescence ratio in saturating [Ca^{2+}] (R_{max}, $1{\cdot}46 \pm 0{\cdot}14$; mean $\pm$ S.E.M., $n = 3$) and zero [Ca^{2+}] (R_{min}, $0{\cdot}19 \pm 0{\cdot}006$) were established. Fluorescence ratios obtained during an experiment could then

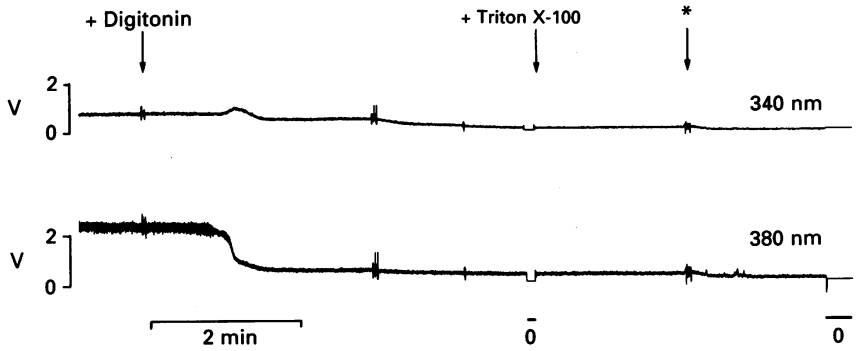

Fig. 3. The effect of digitonin and Triton X-100 on Fura-2 fluorescence recorded from a ventricular myocyte loaded with Fura-2. Traces show fluorescence at 510 nm in response to illumination with 340 nm light (top) and 380 nm light (bottom). Superfusion was stopped and 12 μmol l^{-1} digitonin was added to the cell superfusate where indicated. Flow was restarted after ~ 3 min. Triton X-100 (5%) was subsequently added to the superfusate, where indicated (* indicates where the cell was dislodged upon restarting flow after Triton X-100 addition). Periods marked '0' below the records indicate when a shutter was closed in front of the photomultiplier tube.

be converted into Ca_i^{2+} using the method of Grynkiewicz *et al.* (1985) assuming a K_d of 200 nm (Beuckelmann & Wier, 1988; Williams, Fogarty, Tsien & Fay, 1985). However, it should be noted that the dissociation constant of the dye for Ca^{2+} is affected by Fura-2 binding to intracellular myoplasmic proteins (e.g. Konishi, Hollingworth & Baylor 1988). Thus, the K_d may vary from cell to cell, depending on the extent of intracellular Fura-2 binding and this may alter the absolute value of Ca_i^{2+} calculated by this method.

A second problem is that Fura-2 may be compartmentalized within the cell (Steinberg, Bilezikian & Al-Awqati, 1987), so that the recorded fluorescence signals contain components from non-cytoplasmic sources, and would, therefore, not be an accurate estimate of cytoplasmic [Ca^{2+}]. The experiment shown in Fig. 3 was designed to assess the extent of compartmentation of Fura-2 within intracellular organelles. Cells were treated with 12 μmol l^{-1} digitonin, a concentration sufficient to permeabilize the cell membrane, while leaving the membranes of the intracellular organelles intact (cf. 20 μmol l^{-1} used by Fry, Powell, Twist & Ward, 1984). Figure 3 shows that when 12 μmol l^{-1} digitonin was added to the superfusate there was a rapid loss of fluorescence from the cell. Subsequent addition of 5% Triton X-100 (to break down intracellular membranes) did not produce any further detectable decrease in fluorescence. This result suggests that with our loading conditions there is very little compartmentation of Fura-2 within intracellular organelles. This is supported by the observation that the values of Ca_i^{2+} that we calculate are similar to those reported by Cannell, Berlin & Lederer (1987) who used the free acid of Fura-2, which will not enter non-cytoplasmic compartments.

A third problem that should be considered is that of fluorescence from sources other than the intracellular Fura-2 (e.g. cell autofluorescence, fluorescence from Fura-2 leaking into the bath from other cells and fluorescence from other cells which, although not in the area 'seen' by the photomultiplier tube, may contribute 'stray' fluorescence to the signal). We have estimated the contribution of these sources by monitoring fluorescence from unloaded cells, and fluorescence from the chamber after a Fura-2-loaded cell was moved out of the window monitored by the photomultiplier tube at the end of an experiment. Although these signals were measured and taken into account, they were small and contributed $< 10\%$ to the diastolic cell fluorescence.

Thus although there are problems with calibration, it is possible to obtain a reasonable estimate

of Ca_i^{2+} in these cells. In the present study diastolic Ca_i^{2+} was estimated by taking the average Ca_i^{2+} during the 25 ms before the stimulus and systolic Ca_i^{2+} was estimated by averaging Ca_i^{2+} during 25 ms at the peak of the Ca_i^{2+} transient (cf. Beukelmann & Weir, 1988).

A final potential problem with the use of Fura-2 is that it may act as a Ca^{2+} buffer (Noble & Powell, 1990), which will decrease the free intracellular [Ca^{2+}] during a twitch, and slow the rate of decline of the Ca_i^{2+} transient. We have investigated this possibility by comparing the twitch of unloaded cells with the twitch of cells loaded with Fura-2. The time to peak of the contraction was increased from $144 \pm 5{\cdot}5$ ms ($n = 13$) in unloaded cells to $164 \pm 6{\cdot}5$ ms ($n = 13$; $P = 0{\cdot}025$) in cells loaded with Fura-2, although the half-time of relaxation was not significantly affected by loading with Fura-2. The size of the twitch was reduced significantly ($P = 0{\cdot}03$) from $12{\cdot}1 \pm 0{\cdot}6\%$ ($n = 13$) of cell length in unloaded cells to $9{\cdot}2 \pm 1\%$ ($n = 13$) in Fura-2 loaded cells. Thus it appears that Fura-2 does have some buffering effects, but these are relatively small. From the modelling study of Noble & Powell (1990), we estimate that intracellular [Fura-2] was approximately 70 μmol l^{-1}. In addition, the dye-loading protocol that we used was similar to Wier, Cannell, Berlin, Marban & Lederer (1987) who estimated that intracellular [Fura-2] was approximately 50–100 μmol l^{-1}.

Solutions

The composition of PSS was (in mmol l^{-1}): Na^+, 130·4; Cl^- 142·4; K^+, 5·4; HEPES, 5; glucose, 10; H_2PO_4, 0·4; Mg^{2+}, 3·5; taurine, 20; creatine, 10; Ca^{2+}, 0·75; set to pH 7·2 with NaOH. The control solution used in these experiments contained (mmol l^{-1}): Na^+, 135; K^+, 5; Mg^{2+}, 1; HCO_3^{2-}, 20; Cl^-, 102; SO_4^{2-}, 1; Ca^{2+}, 1; acetate, 20; glucose, 10; insulin, 5 U l^{-1}. This solution was equilibrated with 5% CO_2–95% O_2 to give a pH of 7·3. Ca_o^{2+} was altered by addition of 1 mol l^{-1} $CaCl_2$ to the superfusate. Caffeine was dissolved in the control solution (above) just before use, and ryanodine was kept as a concentrated stock solution which was added to the superfusate just before use. Neither caffeine (10 mmol l^{-1}) nor ryanodine (1 μmol l^{-1}) affected Fura-2 fluorescence *in vitro* at the excitation and emission wavelengths used in the present study.

The solution used to metabolically inhibit the cells before addition of ionomycin for calibration (see above) was made up of the PSS containing Ca^{2+}, 1 mmol l^{-1}, rotenone, 5 μmol l^{-1}, and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), 5 μmol l^{-1}.

Statistics

All data are expressed as means ± S.E.M. of n preparations. Statistical comparisons were made using either a paired t test or Student's t test as appropriate.

RESULTS

The effect of changing stimulation rate on Ca_i^{2+}

Figure 4 shows a slow time-base recording of the effect of changing stimulation rate on cell contraction and the fluorescence ratio (a function of Ca_i^{2+}) in a representative cell. An increase in stimulation rate resulted in a graded increase in cell contraction. This increase in contraction with an increase of stimulation rate was observed in approximately 60% of myocytes. The data in the present paper are taken from such cells. The remaining 40% showed either no change or a decrease in contraction as stimulation rate was increased (cf. Capogrossi, Kort, Spurgeon & Lakatta, 1986). Figure 4 also shows that increasing stimulation rate resulted in a graded increase in both diastolic and peak systolic fluorescence ratios. These changes in Ca_i^{2+} accompanying a change in stimulation rate are more clearly illustrated in Fig. 5*A*, which shows averaged fast time-base records of Ca_i^{2+} and cell length from a cell in which Fura-2 fluorescence was calibrated. At a stimulation frequency of 1 Hz, diastolic Ca_i^{2+} was approximately 70 nmol l^{-1} and the peak of the Ca^{2+} transient (750 nmol l^{-1}) was reached within 50 ms, in good agreement with previous studies using the free acid of Fura-2 to monitor Ca_i^{2+} (Cannell *et al.* 1987). An increase of stimulation frequency from 0·2 to 2 Hz resulted in graded increases of diastolic Ca_i^{2+}

(Harding *et al.* 1989), of the peak of the Ca_i^{2+} transient (Allen & Kurihara, 1980) and in the rate of decline of the Ca_i^{2+} transient (the half-time of decline of the transient decreased significantly from 191 ± 10 ms at 0·2 Hz to 144 ± 12 ms at 1 Hz and 124 ± 10 ms at 2 Hz; $n = 8$). Figure 5*A* also shows that these changes in Ca_i^{2+}

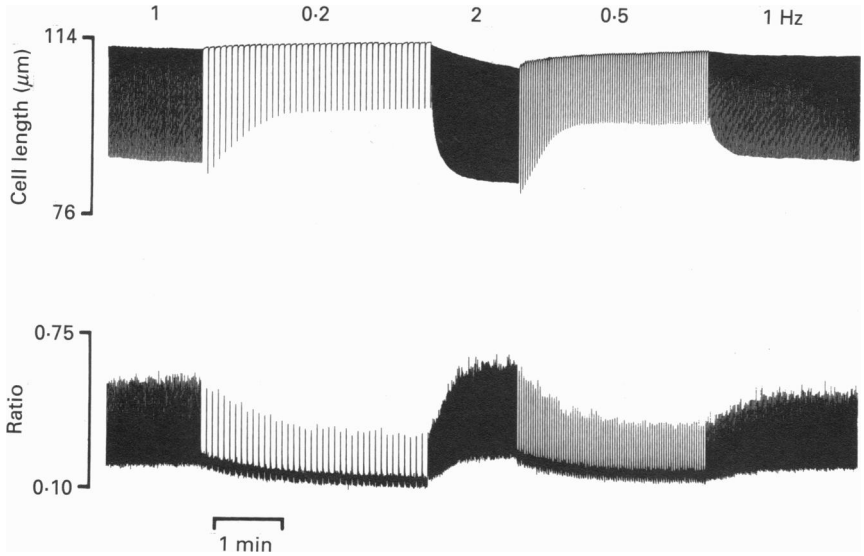

Fig. 4. Slow time-base record of cell length (top trace; contraction is shown as a downward deflection of the cell length trace) and Fura-2 fluorescence ratio (bottom trace) of an isolated rat ventricular myocyte during changes of stimulation rate as indicated above the records.

correlate with a graded decrease in diastolic cell length, an increase in cell contraction and an increase in the rate of twitch relaxation respectively.

Figure 5*B* illustrates the relationship between the estimates of diastolic and systolic Ca_i^{2+} during changes in stimulation rate in this cell. It is apparent that there is a close positive correlation between the increases of systolic and diastolic Ca_i^{2+} observed on changing stimulation rate. Mean diastolic Ca_i^{2+} rose significantly from $70 \pm 18{\cdot}1$ nmol l^{-1} at 0·2 Hz, to $94 \pm 16{\cdot}1$ nmol l^{-1} at 1 Hz to $123 \pm 12{\cdot}8$ nmol l^{-1} at 2 Hz ($n = 7$). The corresponding values for systolic Ca_i^{2+} were 547 ± 72 nmol l^{-1} at 0·2 Hz, 764 ± 112 nmol l^{-1} at 1 Hz and 1030 ± 176 nmol l^{-1} at 2 Hz. The slope of the relationship (using a linear fit) of mean systolic Ca_i^{2+} *vs.* mean diastolic Ca_i^{2+} was 9·1.

The data shown in Fig. 5 support the idea that changes in systolic Ca_i^{2+} accompany changes of diastolic Ca_i^{2+} in the steady state. In order to determine whether this relationship was also present during the non-steady-state phases when stimulation rate was changed (i.e. *throughout* an experiment of the sort shown in Fig. 4), the diastolic fluorescence ratio and the systolic fluorescence ratio were measured continuously during a sequence of rate changes. Figure 6*A* shows changes of systolic and diastolic fluorescence monitored in this way. Figure 6*B* shows a plot of systolic *vs.* diastolic fluorescence ratios throughout this experiment. It is clear that there is a marked positive relationship between the two variables throughout this series of rate

changes. In some experiments, a few points fell above or below the main scatter of points. These outlying points were predominantly from the first few beats after a change of simulation rate, and suggest that other factors may alter the relationship between systolic and diastolic Ca_i^{2+} during these beats (see Discussion).

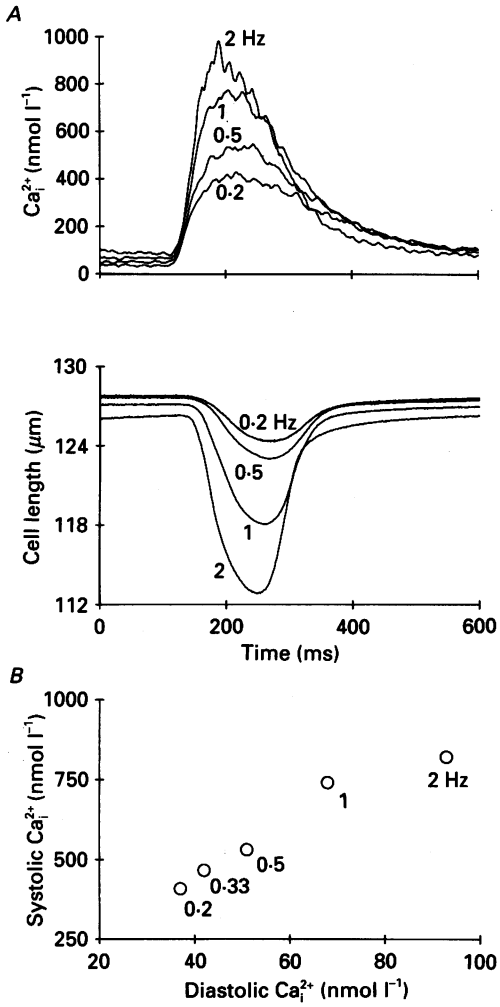

Fig. 5. *A*, fast time-base records of averaged ($n = 16$) Ca_i^{2+} transients (top) and contractions (below) from a rat ventricular myocyte at different stimulation frequencies, as indicated next to each trace. *B*, the relationship between diastolic and systolic Ca_i^{2+} during changes in stimulation rate.

The role of the SR in the observed changes was investigated using the inhibitor ryanodine (1 μmol l^{-1}). The addition of ryanodine to the superfusate resulted in a slow decline in the size of the Ca_i^{2+} transient that was accompanied by a parallel decrease in the size of the contraction to $20 \pm 4\%$ of control (1 Hz, $n = 6$). In addition, a marked increase in diastolic Ca_i^{2+} was observed (cf. Hansford & Lakatta, 1987).

Figure 7*A* shows the effect of changing stimulation rate in the presence of ryanodine. The left panel shows the effect of changing the rate of stimulation from 0·2 to 0·5 Hz on contraction and the fluorescence ratio under control conditions (1 mmol l^{-1} Ca_o^{2+}). The right panel shows the effect of changing stimulation rate in the

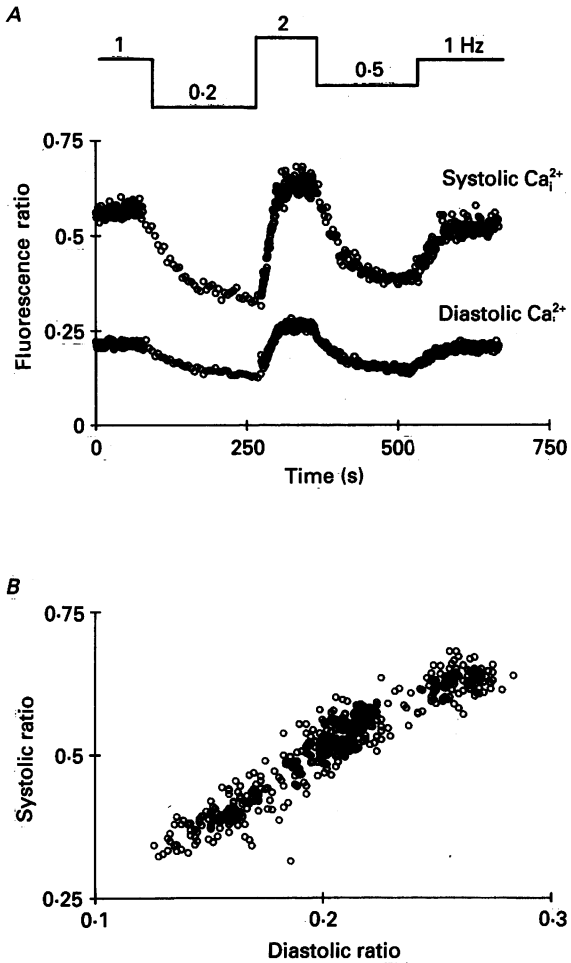

Fig. 6. *A*, changes of systolic and diastolic fluorescence ratio monitored continuously during changes of stimulation rate as indicated above the plots. *B*, systolic *vs.* diastolic fluorescence from the data points shown in *A*.

presence of ryanodine (1 μmol l^{-1}). Ryanodine markedly decreases the changes in the size of the Ca_i^{2+} transient and contraction observed when stimulation rate is changed: systolic Ca_i^{2+} was 207 ± 25 nmol l^{-1} at 0·33 Hz and 223 ± 27 nmol l^{-1} at 2 Hz ($n = 4$). However, the changes in diastolic Ca_i^{2+} observed under control conditions still occurred: diastolic Ca_i^{2+} increased from 160 ± 35 nmol l^{-1} at 0·33 Hz to 193 ± 32 nmol l^{-1} at 2 Hz ($n = 4$). Figure 7*B* shows the effect of ryanodine on the relationship between diastolic Ca_i^{2+} and systolic Ca_i^{2+}. In the presence of 1 μmol l^{-1} ryanodine, there is still a graded increase in diastolic Ca_i^{2+} with changes in rate but

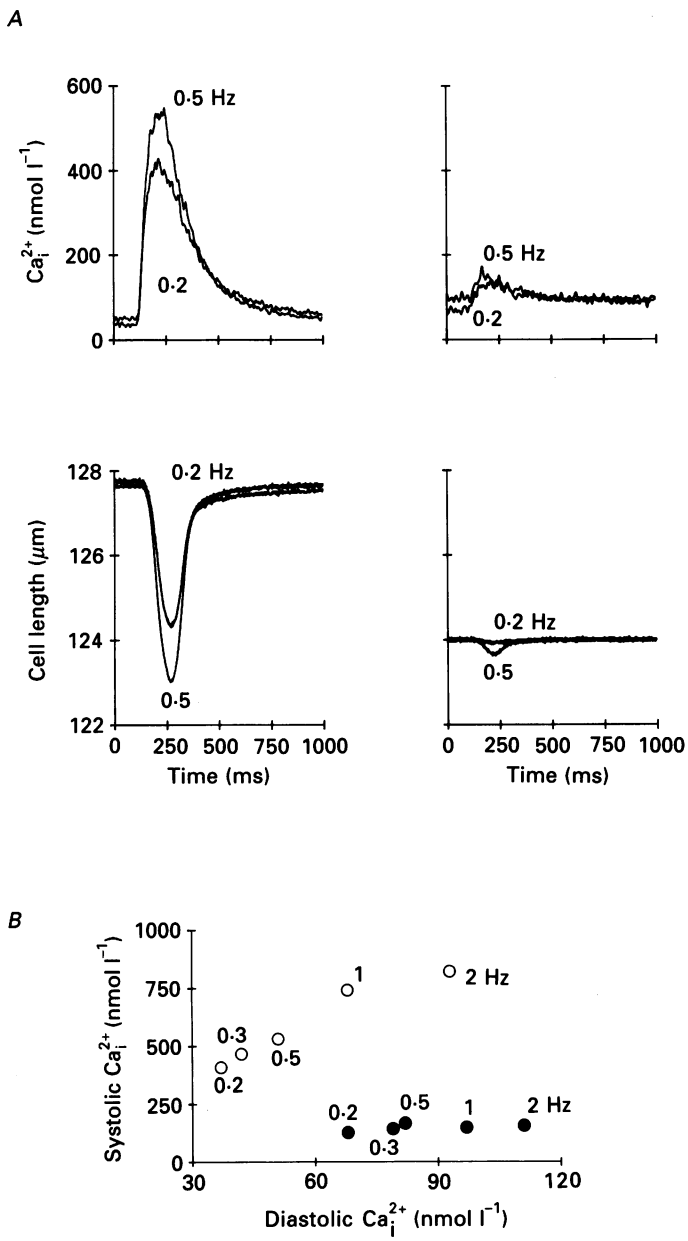

Fig. 7. *A*, the effect of increasing stimulation rate from 0·2 to 0·5 Hz on Ca_i^{2+} (upper panels) and twitch contractions (lower panels) in the absence (left) and presence (right) of 1 μmol l^{-1} ryanodine. *B*, the effect of 1 μmol l^{-1} ryanodine on the relationship between diastolic and systolic Ca_i^{2+} during an increase in stimulation rate in a ventricular myocyte. The points show the relationship in a representative cell in control conditions (○) and in the presence of ryanodine (●) at the stimulation frequencies indicated.

no corresponding increase in the peak systolic Ca_i^{2+}. Hence the slope of the relationship is decreased to 0·41 in the presence of ryanodine.

It appears, therefore, that the changes in the size of the Ca_i^{2+} transient observed during changes of stimulation rate are dependent upon a functional SR. To

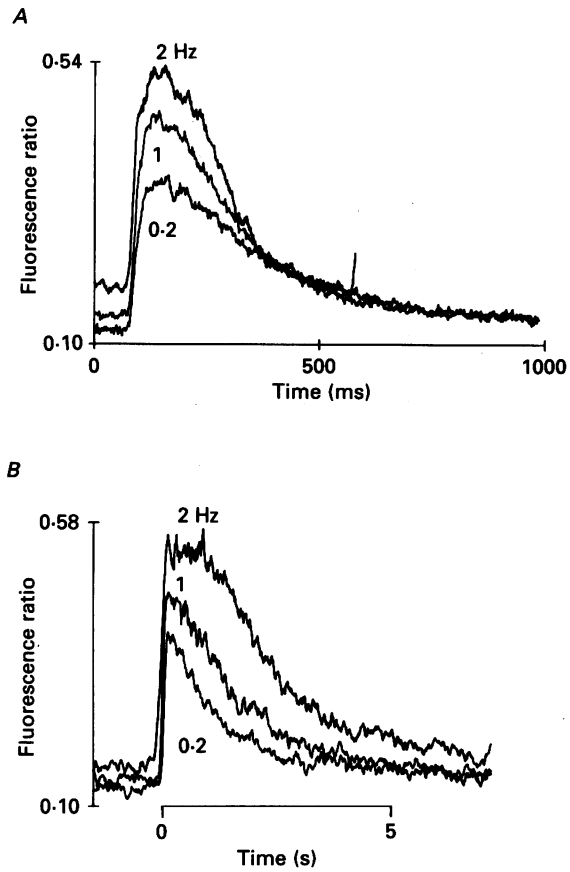

Fig. 8. The effect of a change in stimulation rate on the size of the caffeine-induced increase of Ca_i^{2+}. *A*, averaged ($n = 16$) Fura-2 fluorescence transients from a ventricular myocyte at the stimulation rates indicated. *B*, caffeine-induced increases in Ca_i^{2+} following a post-stimulation rest of 3–5 s. The records in *B* have been superimposed for clarity. The superfusate was changed to the caffeine-containing solution (10 mmol l^{-1} caffeine) immediately after the last stimulus, which occurred approximately 2–3 s before the beginning of the trace. It is difficult to know exactly when the caffeine-containing solution reached the myocyte. However, we estimate that due to tubing dead space and the position of the myocyte within the chamber, there would be a lag of at least 3 s before the caffeine-containing solution reached the cell.

investigate whether these SR-mediated changes in the size of the Ca_i^{2+} transient are associated with changes in Ca^{2+} load of the SR we have used caffeine to rapidly release Ca^{2+} from the SR (cf. Smith *et al.* 1988).

Cells were stimulated at a given frequency until contraction was in a steady state. Stimulation was then stopped, and ~ 2 s later, 10 mmol l^{-1} caffeine was applied to

the cell. Figure 8*B* illustrates the caffeine-induced increase in Ca_i^{2+} recorded from a cell (in 1 mmol l^{-1} Ca_o^{2+}) following trains of stimuli at 1, 2 and 0·2 Hz. The amplitude of the caffeine-induced increase of Ca_i^{2+} is increased when stimulation rate is increased, and this increase correlates with the observed increases in diastolic and systolic Ca_i^{2+} (Fig. 8*A*). Similar results were obtained in four other cells.

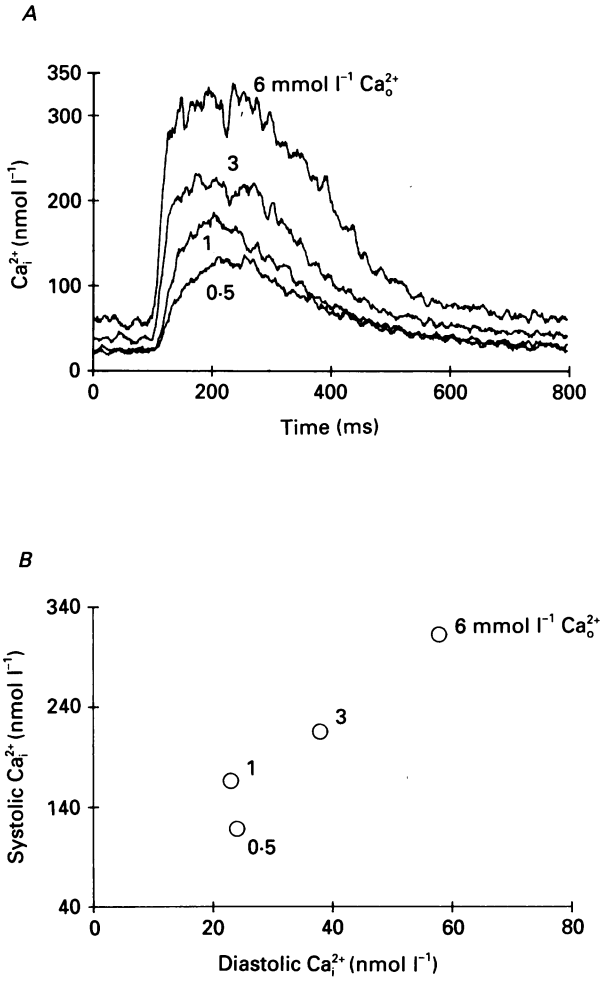

Fig. 9. *A*, averaged (n = 16) Ca_i^{2+} transients showing the effect of increasing Ca_o^{2+} on Ca_i^{2+} during stimulation at 1 Hz. *B*, the effect of increasing Ca_o^{2+} upon the relationship between diastolic and systolic Ca_i^{2+} from the cell in *A*.

The effect of changing Ca_o^{2+} on Ca_i^{2+}

Figure 9*A* shows fast time-base, averaged recordings of Ca_i^{2+} as Ca_o^{2+} was increased from 0·5 to 6 mmol l^{-1} at a constant stimulation rate of 1 Hz. As Ca_o^{2+} was increased, there was a graded increase in both diastolic Ca_i^{2+} and in peak systolic Ca_i^{2+} and a small decrease in the rate of decline of the Ca_i^{2+} transient (the half-time of decline of the Ca_i^{2+} transient increased from 151 ± 6·4 ms in 1 mmol l^{-1} Ca_o^{2+} to 168 ± 12·5 ms in

6 mmol l^{-1} Ca_o^{2+}; $n = 7$). These changes of Ca_i^{2+} were accompanied by a decrease in resting cell length, an increase in cell contraction (not shown, but see Fig. 10*A*), and a small slowing in the rate of relaxation of the twitch (not shown). Figure 9*B* shows the relationship between diastolic Ca_i^{2+} and systolic Ca_i^{2+}, as Ca_o^{2+} was increased, in

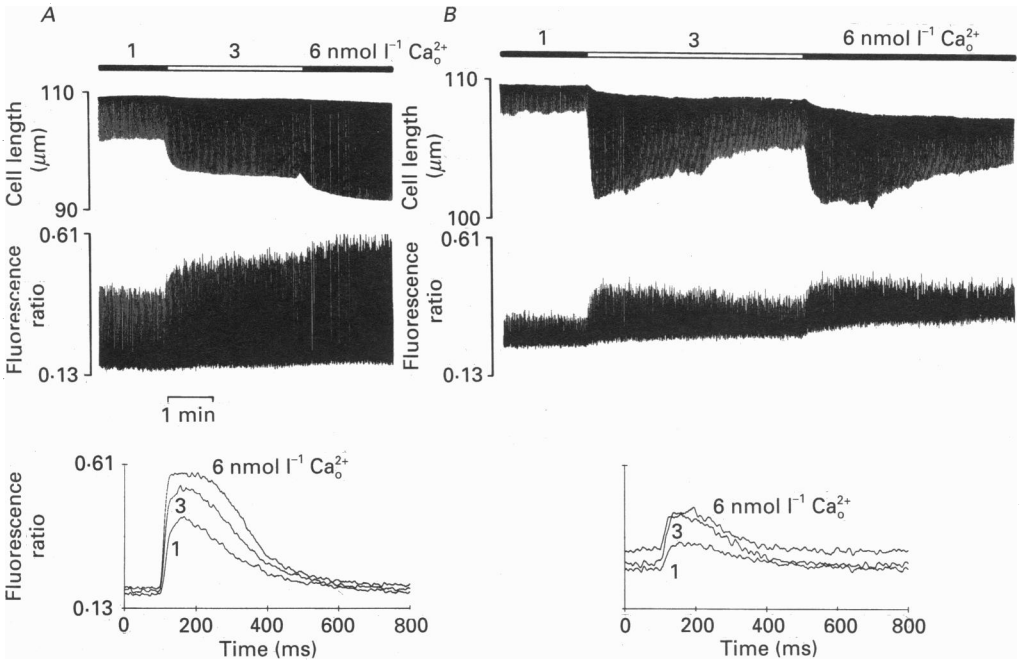

Fig. 10. *A*: upper panel, slow time-base record showing the effect of increasing Ca_o^{2+} on cell length (top trace) and Fura-2 fluorescence ratio (bottom trace) during stimulation at 1 Hz; lower panel, averaged ($n = 16$) Fura-2 fluorescence transients at each Ca_o^{2+}. *B*: upper panel, the effect of increasing Ca_o^{2+} in the same cell following 20 min superfusion with 1 μmol l^{-1} ryanodine (note the change in cell length scale bar); lower panel, Fura-2 fluorescence transients averaged ($n = 16$) during the peak of the Ca_i^{2+} response to Ca_o^{2+} in the presence of ryanodine.

this cell. At a stimulation rate of 1 Hz, diastolic Ca_i^{2+} increased from 65 ± 9 nmol l^{-1} ($n = 5$; $Ca_o^{2+} = 0{\cdot}5$ mmol l^{-1}) to 106 ± 17 nmol l^{-1} ($Ca_o^{2+} = 6$ mmol l^{-1}) while systolic Ca_i^{2+} increased from 350 ± 49 to 770 ± 63 nmol l^{-1}. Thus the relationship between systolic and diastolic Ca_i^{2+} appeared similar to that during changes in stimulation rate.

We have, therefore, investigated the role of the SR in the response to increasing Ca_o^{2+}. Figure 10 shows the effect of 1 μmol l^{-1} ryanodine on the response of a myocyte to changes in Ca_o^{2+}. Under control conditions, raising Ca_o^{2+} from 1 to 3 and finally to 6 mmol l^{-1} produced graded and sustained increases in cell contraction (upper panel, top trace) which were accompanied by graded increases in both the diastolic and peak systolic ratios. However, when Ca_o^{2+} was increased in the presence of ryanodine there was a biphasic response; cell contraction increased rapidly and subsequently declined. This was accompanied by a marked increase in both the diastolic and peak systolic ratios. The contractile response then declined to a steady level. This decline

in contractility occurred in parallel with a decline in the size of the Ca_i^{2+} transient. However, diastolic Ca_i^{2+} continued to increase. Similar changes were observed in three other cells when Ca_o^{2+} was increased in the presence of ryanodine. This makes plotting the relationship between systolic and diastolic Ca_i^{2+} in the presence of ryanodine

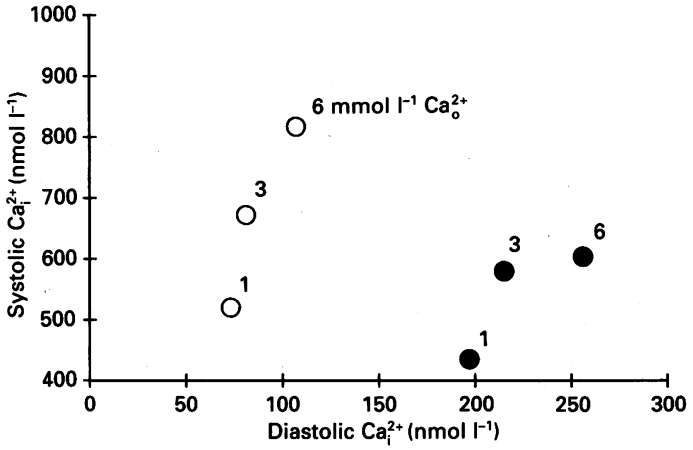

Fig. 11. The effect of 1 μmol l^{-1} ryanodine (●) on the relationship between diastolic and systolic Ca_i^{2+} during changes in Ca_o^{2+} (control shown as ○), from an experiment such as that illustrated in Fig. 10.

difficult, as the relationship will vary with time after exposure to the higher Ca_o^{2+}. However, Fig. 11 illustrates the effect of ryanodine on the relationship between diastolic and peak systolic Ca_i^{2+} at the peak Ca_i^{2+} after the increase of Ca_o^{2+}. Although, in the presence of ryanodine, raising Ca_o^{2+} still produced a large graded increase in diastolic Ca_i^{2+}, the accompanying increase in peak systolic Ca_i^{2+} was very much reduced compared with control. The slope of the relationship between systolic and diastolic Ca_i^{2+} was depressed only slightly. However, after a longer exposure to high Ca_o^{2+}, systolic Ca_i^{2+} decreased towards the level in 1 mmol l^{-1} Ca_o^{2+} (Fig. 10), so that the relationship between systolic and diastolic Ca_i^{2+} became flatter after a longer exposure to increased Ca_o^{2+}.

To investigate further the role of the SR when Ca_o^{2+} is increased we have used caffeine to stimulate Ca^{2+} release from the SR. Figure 12*A* shows Ca_i^{2+} transients, and a subsequent caffeine-induced increase of Ca_i^{2+} recorded in 1 mmol l^{-1} Ca_o^{2+} (stimulation rate 1 Hz), while Fig. 12*B* shows the Ca_i^{2+} transients and the caffeine-induced increase of Ca_i^{2+} recorded in the same cell after Ca_o^{2+} had been increased to 6 mmol l^{-1}: increasing Ca_o^{2+} led to an increase in diastolic Ca_i^{2+} systolic Ca_i^{2+}, and the amount of Ca^{2+} that could be released by caffeine.

The effect of changes in stimulation rate when Ca_o^{2+} is altered

Because of the qualitative similarity of the relationships between systolic and diastolic Ca_i^{2+} during changes of stimulation rate (Fig. 5*B*) and changes of Ca_o^{2+} (Fig. 9*B*) we were interested to see whether there was also a quantitative similarity. We investigated, therefore, the relationship between systolic and diastolic Ca_i^{2+} when stimulation rate was altered at different Ca_o^{2+}. Figure 13*A* shows slow time-base recordings of Fura-2 fluorescence when stimulation rate was altered at 1 mmol l^{-1}

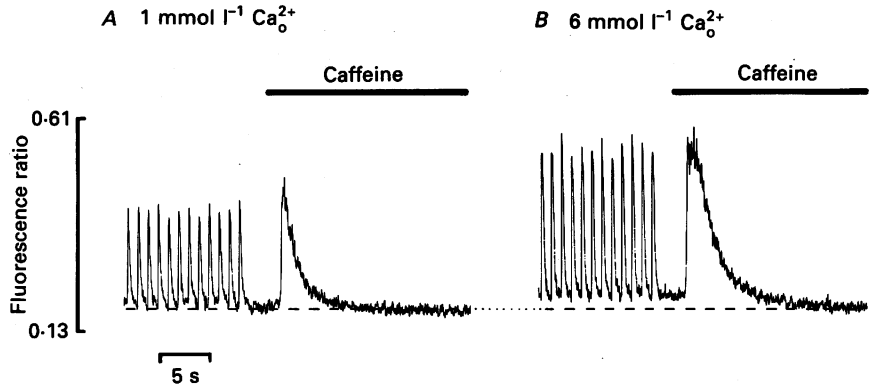

Fig. 12. The effect of increasing Ca_o^{2+} on the caffeine-releasable pool of Ca^{2+}. Fura-2 fluorescence was recorded during stimulation at 1 Hz, and during the application of 10 mmol l^{-1} caffeine (bar) approximately 4 s after stopping stimulation, in 1 mmol l^{-1} Ca_o^{2+} (*A*) and in 6 mmol l^{-1} Ca_o^{2+} (*B*).

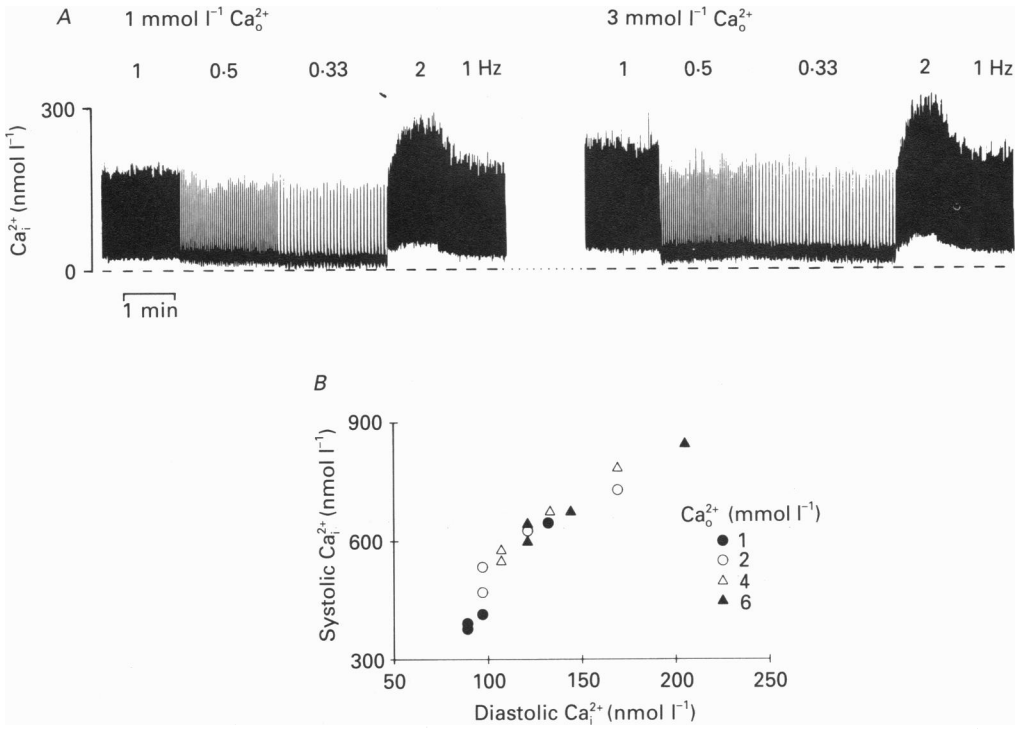

Fig. 13. *A*, slow time-base record showing the effect of changes in stimulation rate on Ca_i^{2+} in 1 mmol l^{-1} Ca_o^{2+} (left) and 3 mmol l^{-1} Ca_o^{2+} (right). *B*, the relationship between diastolic and systolic Ca_i^{2+} during changes in stimulation rate at different Ca_o^{2+}. Each symbol represents a different stimulation frequency between 0·3 and 2 Hz in the Ca_o^{2+} as indicated.

Ca_o^{2+} and 3 mmol l^{-1} Ca_o^{2+}. It shows qualitatively similar changes to those described previously: increasing either Ca_o^{2+} or stimulation rate increased both systolic and diastolic Ca_i^{2+}. Figure 13*B* shows the relationship between diastolic and

systolic Ca_i^{2+} from a similar experiment. It is clear that when stimulation rate is altered between 0·3 and 2 Hz at Ca_o^{2+} between 1 and 6 mmol l^{-1}, there is a unique relationship between systolic and diastolic Ca_i^{2+}. However, when Ca_o^{2+} was lowered to 0·5 mmol l^{-1}, the curve was depressed, so that at a given diastolic Ca_i^{2+}, systolic Ca_i^{2+} was lower (approximately 50%) than that observed at higher Ca_o^{2+} (not shown).

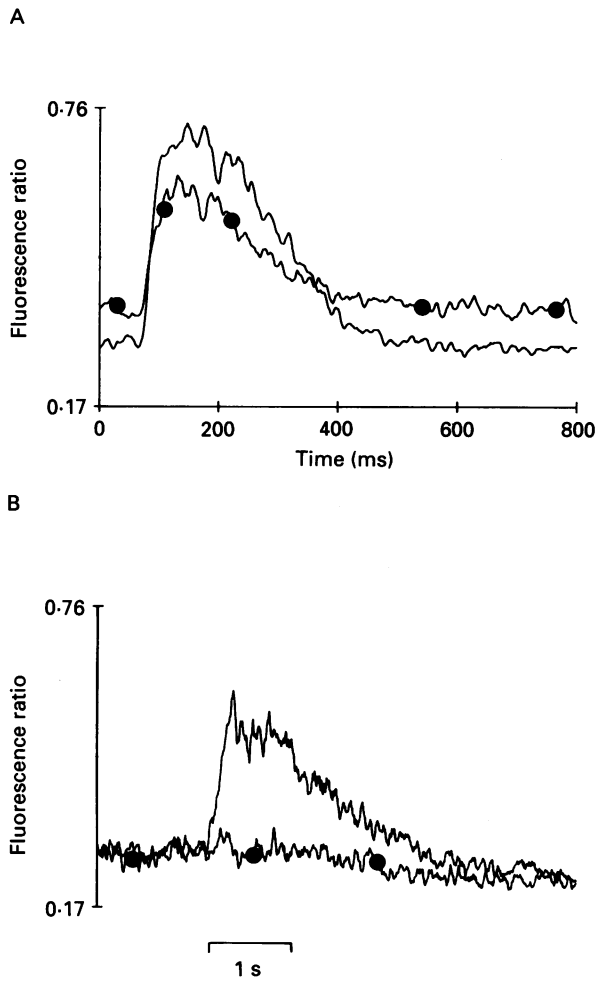

Fig. 14. The effect of 1 μmol l^{-1} ryanodine on the caffeine-releasable pool of Ca^{2+}. *A*, averaged ($n = 16$) Ca_i^{2+} transients recorded in control (1 Hz, 6 mmol l^{-1} Ca_o^{2+}) and after 20 min superfusion with 1 μmol l^{-1} ryanodine (indicated by ●). *B*, the corresponding caffeine-induced increases in Ca_i^{2+}.

The effect of ryanodine on caffeine-induced Ca^{2+} release

Our interpretation of the experiments in which ryanodine was used depends upon the assumption that the SR is functionally inhibited by ryanodine (Sutko, Ito & Kenyon, 1985; Bers, Bridge & MacLeod, 1987). We have, therefore, used caffeine to assess the Ca^{2+} load of the SR in the presence of ryanodine. Figure 14 illustrates Ca_i^{2+}

transients and caffeine-induced increases in Ca_i^{2+} recorded in 6 mmol l^{-1} Ca_o^{2+} before and after 20 mins superfusion with 1 μmol l^{-1} ryanodine. Figure 14*A* shows the increase in the diastolic fluorescence ratio (Hansford & Lakatta, 1987) and the decrease in the peak systolic fluorescence ratio (Marban & Wier, 1985) produced by ryanodine. Figure 14*B* shows that in 6 mmol l^{-1} Ca_o^{2+} in the absence of ryanodine, a large release of Ca^{2+} was obtained using 10 mmol l^{-1} caffeine. However, after 20 mins superfusion with ryanodine virtually no release of Ca^{2+} was obtained on application of caffeine. These results suggest that ryanodine inhibits the normal Ca^{2+}-handling ability of the SR and that the large increase in Ca_i^{2+} observed with caffeine results exclusively from the release of Ca^{2+} from the SR and not from Ca^{2+} entry from the extracellular space (also supported by the observation that if the caffeine-containing solution is nominally Ca^{2+} free, the caffeine-induced Ca^{2+} release is not affected – not shown).

DISCUSSION

One of the main findings in the present study is that under many circumstances there appears to be a positive correlation between changes in systolic and diastolic Ca_i^{2+} and SR Ca^{2+} content. Before discussing this in more detail, however, the problems associated with the use of isolated myocytes and Fura-2 AM should be addressed.

The use of isolated myocytes

The use of isolated myocytes for studies of cardiac muscle is now well established. The advantage for the present study was the ability to monitor contraction and Ca_i^{2+} in the same cell, and the ability to apply caffeine rapidly, with few diffusion delays, to obtain a large response (cf. Smith *et al.* 1988). However, the majority of cells that we isolate from the ventricles of rat hearts show an increase in contraction as stimulation rate is increased (i.e. a positive force–frequency relationship; see Figs 4–8). This was also observed in cells that had not been loaded with Fura-2. Previous studies using multicellular preparations from rat hearts have usually shown a negative force–frequency relationship (i.e. a decrease in contraction as stimulation rate is increased; Hoffman & Kelly, 1959). There are a number of possible explanations for this discrepancy. First, the cells may have been damaged in some unknown way during the isolation procedure. Although this is possible, it must be very selective damage to produce such a functional change in otherwise viable cells. Second, it is possible that multicellular preparations of rat heart become hypoxic or metabolically depleted (Henry, 1975; Schouten & ter Keurs, 1986) at high stimulation rates, and it is this that leads to the decrease in contractility. We do not think that this is the case because it cannot explain why multicellular preparations from other species show a positive force–frequency relationship. In addition it cannot explain why Ca_i^{2+} transients monitored in superficial cells of rat papillary muscles (which would be unlikely to become hypoxic) also decrease as stimulation rate is increased (Orchard & Lakatta, 1985). Nor can it explain why some of the single cells that we isolate show a negative force–frequency relationship, although they are unlikely to be hypoxic. The third possibility is that the degree of Ca^{2+} loading of the cell determines the force–frequency relationship. Capogrossi *et al.* (1986) have

suggested that cells that are Ca^{2+} loaded have a negative force–frequency relationship. Those that are not Ca^{2+} loaded have a positive force–frequency relationship. Since the cells used in the present study were chosen because they did not exhibit signs of Ca^{2+} loading (e.g. spontaneous contractile activity), it seems that this is a possible explanation for the positive force–frequency relationship observed in the present study. It is also worth noting that Allen & Kurihara (1980) reported that rat papillary muscles showed a negative force–frequency relationship soon after mounting in a muscle bath, but that with time they could start to show a positive force–frequency relationship. This could be due to a decrease in the Ca^{2+} load of cells within the preparation with time after dissection, which may slightly damage the preparation, and hence lead to Ca^{2+} loading.

The use of Fura-2 to monitor Ca_i^{2+}

Many of the problems involved in the use of Fura-2 were discussed in the Methods section. We have attempted to take into account the known problems of quantifying Fura-2 fluorescence, and the values of Ca_i^{2+} that we obtain are in relatively good agreement with previous estimates of both diastolic and systolic Ca_i^{2+} (e.g. Cannell *et al.* 1987). However, in view of other unknowns such as possible binding of Fura-2 to intracellular proteins within the cytoplasm and the K_d of Fura-2 within the cell (see Methods), it is still not possible to determine absolute values of Ca_i^{2+} with any certainty.

The relationship between systolic and diastolic Ca_i^{2+}

The main observation in the present paper is that there is a positive correlation between diastolic and systolic Ca_i^{2+} in isolated single rat ventricular myocytes loaded with Fura-2. A similar correlation between diastolic and systolic Ca_i^{2+} has been previously observed in chick embryonic myocardial cell aggregates loaded with Indo-1 AM (Lee & Clusin, 1987). These observations are consistent with the hypotheses that have been put forward previously to account for how changes of stimulation rate and raising Ca_o^{2+} lead to increases in the size of the Ca_i^{2+} transient: i.e. increasing stimulation rate increases intracellular $[Na^+]$ (by increasing Na^+ influx per unit time) which, via the Na^+–Ca^{2+} exchange mechanism, increases cytoplasmic, and hence SR $[Ca^{2+}]$ (see Introduction). Similarly increasing Ca_o^{2+} will increase Ca^{2+} influx into the cell (via the Ca^{2+} current, Ca^{2+} leak, and Na^+–Ca^{2+} exchanger; Kirby, Orchard & Boyett, 1989), and so elevate cytoplasmic (Sheu & Fozzard, 1982) and hence SR $[Ca^{2+}]$. The increased SR $[Ca^{2+}]$ means that more Ca^{2+} is available for release, leading to a larger Ca_i^{2+} transient.

This hypothesis is supported by several observations in the present study. First, there was a positive correlation between diastolic and systolic Ca_i^{2+} during increases of stimulation rate and Ca_o^{2+}. For changes of stimulation rate this is true both in the steady state (Fig. 5) and in non-steady-state conditions (Fig. 6). In the latter case, although most of the points of the relationship of systolic *vs.* diastolic Ca_i^{2+} lie on a single line (Fig. 6), in some experiments there were some outliers. These came mainly from the first few beats following a change in stimulation rate. This suggests that the size of the first few beats following a change in stimulation rate is not dependent on diastolic Ca_i^{2+} but may depend on other factors, such as the degree of mechanical

restitution (Braveny & Kruta, 1958). However, the subsequent slower changes may depend on diastolic Ca_i^{2+}. Second, the Ca^{2+} load of the SR (assessed using caffeine: Weber & Herz, 1968; Chapman & Leoty, 1976; Smith *et al.* 1988) increased in parallel with diastolic and systolic Ca_i^{2+} during these manoeuvres. Third, inhibition of the SR with ryanodine reduced the observed changes in systolic Ca_i^{2+}, but changes in diastolic Ca_i^{2+} still occurred during changes in stimulation rate.

There are, however, two observations that complicate the interpretation of the present results. First, when Ca_o^{2+} was altered in the presence of ryanodine, systolic Ca_i^{2+} altered biphasically, with an initial increase followed by a decrease to a steady level, while diastolic Ca_i^{2+} increased monotonically (Fig. 10). Under these conditions the Ca_i^{2+} transient presumably reflects mainly Ca^{2+} entry across the cell membrane via the Na^+–Ca^{2+} exchanger and I_{Ca}. The biphasic effect of raising Ca_o^{2+} on the Ca_i^{2+} transient may then be understood as an increased influx of Ca^{2+} on the Na^+–Ca^{2+} exchanger during each action potential in response to an increase in Ca_o^{2+}. However, the increased Ca_o^{2+} will, with time, lead to a decrease of intracellular [Na^+] as Na^+ is extruded on the Na^+–Ca^{2+} exchanger (Deitmer & Ellis, 1978). This will tend to decrease further Ca^{2+} entry on the exchanger (Eisner, Allen & Orchard, 1985) and hence, in the presence of a Ca^{2+} extrusion pathway, will decrease Ca_i^{2+} (cf. Allen, Eisner & Orchard, 1984). In addition, I_{Ca} will initially increase when Ca_o^{2+} is increased (e.g. Kirby *et al.* 1989) leading to a larger Ca_i^{2+} transient. However, I_{Ca} will then decline as diastolic Ca_i^{2+} increases (Boyett, Kirby & Orchard, 1988) tending to produce a secondary decline in the size of the Ca_i^{2+} transient. The monophasic increase in diastolic Ca_i^{2+} is presumably due to an increased Ca^{2+} leak into the cell due to the raised Ca_o^{2+}.

Secondly, the relationship between systolic and diastolic Ca_i^{2+} was different when Ca_o^{2+} was 0·5 mmol l^{-1} than when it was 1 mmol l^{-1} or above; systolic Ca_i^{2+} was lower for a given diastolic Ca_i^{2+}. It appears possible that under these circumstances, the low Ca_o^{2+} may be leading to a decrease in I_{Ca}, and it is this that is limiting Ca^{2+} release from the SR (Fabiato, 1985).

Finally, the hypothesis outlined above assumes that the SR Ca^{2+} load (and hence the Ca_i^{2+} transient) depends only on diastolic cytoplasmic Ca^{2+}. This is a gross simplification for four reasons. Firstly, because it is clear that other factors, such as action potential duration (Morad & Trautwein, 1968; Fabiato, 1985), the magnitude of I_{Ca} (Fabiato, 1985) and the degree of mechanical restitution (Braveny & Kruta, 1958) will also alter Ca^{2+} release from the SR. However, it is intriguing that the relationship between systolic and diastolic Ca_i^{2+} is the same, in a particular cell, during changes of stimulation rate and Ca_o^{2+} (e.g. Fig. 13*B*), which would be expected to alter the action potential, I_{Ca} and the degree of mechanical restitution to different extents. Secondly, it assumes that the SR Ca^{2+} load is a consequence of the level of cytoplasmic Ca^{2+}. An alternative explanation is that the two compartments (cytoplasm and SR) load in parallel, although this assumes that there is a non-cytoplasmic pathway through which the SR can load with Ca^{2+}. Thirdly, it is possible that cytoplasmic Ca^{2+} is determined by the Ca^{2+} content of the SR as a result of Ca^{2+} leak from the SR. This seems unlikely, however, because ryanodine appears to inhibit the changes in SR Ca^{2+} load and yet changes in diastolic Ca_i^{2+} still occur (Fig. 7*B*). Finally, an increase in diastolic Ca_i^{2+} could result in a greater occupation of

intracellular buffer sites for Ca^{2+} (e.g. calmodulin, troponin). Thus, even if the amount of Ca^{2+} released from the SR is unchanged, since less of the released Ca^{2+} will be intracellularly buffered, the peak systolic Ca_i^{2+} will be greater.

The role of the SR in the relationship between systolic and diastolic Ca_i^{2+}

The role of the SR in the responses seen during changes in stimulation rate and Ca_o^{2+} was discussed briefly in the previous section. The conclusions depend, however, on assumptions about the SR inhibitors used.

First, it has been suggested that ryanodine increases the leak of Ca^{2+} from the SR (Hansford & Lakatta, 1987; Meissner, 1986), so that at short interstimulus intervals, some Ca^{2+} may remain in the SR (Bers *et al.* 1987). However, in the presence of ryanodine, the SR may still accumulate Ca^{2+}, but it loses this Ca^{2+} very rapidly at the onset of rest, declining with a half-time of about 1 s (Bers *et al.* 1987). Thus, although in the present study we could not release Ca^{2+} from the SR using caffeine in the presence of ryanodine, the minimum interval after the cessation of stimulation that we could apply caffeine was 3 s (e.g. Fig. 12). Therefore the possibility remains that at intervals of less than 3 s, in the presence of ryanodine, the SR does indeed contain Ca^{2+} that is available for release by caffeine.

Second, the use of caffeine as an agent to release Ca^{2+} from the SR should be considered. The contracture of cardiac muscle produced by caffeine (Chapman & Leoty, 1976) appears to be due to a sensitization of the contractile proteins to Ca^{2+} (Wendt & Stephenson, 1983) and an increase of Ca_i^{2+} (Smith *et al.* 1988), which appears to come predominantly from the SR (Weber & Herz, 1968; Smith *et al.* 1988; present study). Because of this dual action, it is necessary to monitor Ca_i^{2+} to compare the amount of Ca_i^{2+} released from the SR by caffeine with the size of the Ca_i^{2+} transient. However, in ferret papillary muscles the caffeine-induced increase of Ca_i^{2+} is smaller than the Ca_i^{2+} transient (Smith *et al.* 1988), possibly because of the difficulty of ensuring rapid and uniform application of caffeine to a multicellular preparation (Smith *et al.* 1988). This suggestion is supported by the observation that when diffusion delays are minimized by the use of single myocytes, the caffeine-induced increase of Ca_i^{2+} is quantitatively similar to the size of the Ca_i^{2+} transient (present study; O'Neill, Donoso & Eisner, 1990). The other consideration is that it is not clear that caffeine releases Ca^{2+} from the same SR pool as that released by a physiological stimulus, although there does appear to be a good correlation between the size of the Ca_i^{2+} transient released by electrical stimulation and that reached by caffeine (Figs 8 and 12). Thus the present results may imply that the entire Ca^{2+} content is released during the twitch which would be inconsistent with a 'graded' Ca^{2+}-induced Ca^{2+} release mechanism (Fabiato 1983). Furthermore, studies using rapid-cooling contractures to assess SR Ca^{2+} content in isolated rabbit myocytes (e.g. Bers, Bridge & Spitzer 1989) suggest that the SR contains a greater amount of Ca^{2+} than is released by a single action potential. Thus, in studies using the superfusion of caffeine to assess the SR Ca^{2+} content in isolated myocytes, neither caffeine nor the action potential may be releasing all of the Ca^{2+} stored within the SR. Under different conditions, the action potential and caffeine may release a constant fraction of the SR Ca^{2+} load (which changes), or they may be releasing a different fraction of the SR Ca^{2+} load (which stays constant), or a combination of these possibilities. Such similar

actions of caffeine and the action potential on Ca^{2+} release may be because caffeine may work by sensitizing the normal release mechanism to Ca^{2+} (O'Neill & Eisner, 1990), so that Ca^{2+} is released from the SR even at diastolic Ca_i^{2+}.

The results using caffeine and ryanodine when stimulation rate was increased suggested that both the Ca^{2+} load of the SR and Ca^{2+} release from the SR increased as stimulation rate was increased. In the presence of ryanodine, increasing stimulation rate still led to increases in diastolic Ca_i^{2+}, but not systolic Ca_i^{2+} (Fig. 7*B*). Thus the *amplitude* of the Ca_i^{2+} transient (i.e. systolic − diastolic Ca_i^{2+}) decreased as stimulation rate was increased. This may be because I_{Ca} decreases when stimulation rate is increased (Mitchell, Powell, Terrar & Twist, 1985; Fedida, Noble & Spindler, 1988), possibly as a consequence of the increase in diastolic Ca_i^{2+} (Mitchell *et al.* 1985; Boyett *et al.* 1988). In addition, shortening of the action potential (Mitchell *et al.* 1985; Fedida *et al.* 1988) and the increase of diastolic Ca_i^{2+} would decrease Ca^{2+} entry on Na^+–Ca^{2+} exchange (Kirby *et al.* 1989). Both of these changes would, therefore, tend to decrease the size of the Ca_i^{2+} transient from non-SR sources in the presence of ryanodine. The observation of a flat force–frequency relationship in the presence of ryanodine contrasts with previous studies (e.g. Sutko & Willerson, 1980; Bers, 1985) which have reported a positive force–frequency relationship in the presence of ryanodine in rat ventricular muscle. However, a flat force–frequency relationship may be partially explained in the ryanodine-treated SR is still able to accumulate Ca^{2+} and hence transiently buffer rapid Ca_i^{2+} changes in the cytoplasm (e.g. Bers *et al.* 1987). Thus, a significant fraction of the Ca^{2+} entering the cell during the action potential may be absorbed by the SR and hence not activate additional force.

Conversely, when Ca_o^{2+} was increased in the presence of ryanodine, both diastolic and systolic Ca_i^{2+} still increased. It seems likely that this may be because I_{Ca}, and Ca^{2+} influx on the Na^+–Ca^{2+} exchange mechanism, will both increase as Ca_o^{2+} is increased, and will therefore contribute to the increase in the size of the calcium transient as Ca_o^{2+} is increased.

Conclusion

The results in the present study show that raising Ca_o^{2+} produces increases in both diastolic and systolic Ca_i^{2+}, measured in isolated rat ventricular myocytes using Fura-2 AM. Similar changes in Ca_i^{2+} were also observed in rat ventricular myocytes which responded to an increase in stimulation rate with an increase in contraction. Changes in diastolic and systolic Ca_i^{2+} appear closely related over a wide range of experimental conditions, although this relationship is altered by the SR inhibitor ryanodine. Experiments in which caffeine was used to assess the SR Ca^{2+} content suggested that an increase in diastolic Ca_i^{2+} is accompanied by an increase in the Ca^{2+} available for release from the SR. These results are consistent with a change in diastolic Ca_i^{2+} leading to an increase in the Ca^{2+} content of the SR and hence an increase in the size of the Ca_i^{2+} transient.

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REFERENCES

ALLEN, D. G., EISNER, D. A. & ORCHARD, C. H. (1984). Factors influencing free intracellular calcium concentration in quiescent ferret ventricular muscle. *Journal of Physiology* **350**, 615–630.

ALLEN, D. G. & KURIHARA, S. (1980). Calcium transients in mammalian ventricular tissue. *European Heart Journal* **1**, suppl. A, 5–15.

BERS, D. M. (1985). Ca influx and sarcoplasmic reticulum Ca release in cardiac muscle activation during postrest recovery. *American Journal of Physiology* **248**, H366–381.

BERS, D. M., BRIDGE, J. H. B. & MACLEOD, K. T. (1987). The mechanism of ryanodine action in rabbit ventricular muscle evaluated with Ca-selective microelectrodes and rapid cooling contractures. *Canadian Journal of Physiology and Pharmacology* **65**, 610–618.

BERS, D. M., BRIDGE, J. H. B. & SPITZER, K. W. (1989). Intracellular Ca^{2+} transients during rapid cooling contractures in guinea-pig ventricular myocytes. *Journal of Physiology* **417**, 537–553.

BEUCKELMANN, D. J. & WIER, W. G. (1988). Mechanism of release of calcium from sarcoplasmic reticulum of guinea-pig cardiac cells. *Journal of Physiology* **405**, 233–255.

BOUCHARD, R. A. & BOSE, D. (1989). Analysis of the interval–force relationship in rat and canine ventricular myocardium. *American Journal of Physiology* **257**, H2036–2047.

BOYETT, M. R., HART, G., LEVI, A. J. & ROBERTS, A. (1987). Effects of repetitive activity on developed force and intracellular sodium activity in isolated sheep and dog Purkinje fibres. *Journal of Physiology* **388**, 295–322.

BOYETT, M. R., KIRBY, M. S. & ORCHARD, C. H. (1988). Rapid regulation of the 'second inward current' by intracellular calcium in isolated rat and ferret ventricular myocytes. *Journal of Physiology* **407**, 77–102.

BOYETT, M. R., MOORE, M., JEWELL, B. R., MONTGOMERY, R. A. P., KIRBY, M. S. & ORCHARD, C. H. (1988). An improved apparatus for the optical recording of contraction of single heart cells. *Pflügers Archiv* **413**, 197–205.

BRAVENY, P. & KRUTA, V. (1958). Dissociation de deux facteurs: restitution et potentiation dans l'action de l'intervalle sur l'amplitude de la contraction du myocarde. *Archives Internationales de Physiologie et de Biochimie* **66**, 633–652.

CANNELL, M. B., BERLIN, J. R. & LEDERER, W. J. (1987). Effect of membrane potential changes on the calcium transient in single rat cardiac cells. *Science* **238**, 1419–1423.

CANNELL, M. B. & LEDERER, W. J. (1986). A novel experimental chamber for single cell voltage-clamp and patch-clamp applications with low electrical noise and excellent flow and temperature control. *Pflügers Archiv* **406**, 536–539.

CAPOGROSSI, M. C., KORT, A. A., SPURGEON, H. A. & LAKATTA, E. G. (1986). Single adult rabbit and rat myocytes retain the Ca^{2+}- and species-dependent systolic and diastolic contractile properties of intact muscle. *Journal of General Physiology* **88**, 589–613.

CHAPMAN, R. A. & LEOTY, C. (1976). The time-dependent and dose-dependent effects of caffeine on the contraction of the ferret heart. *Journal of Physiology* **256**, 287–314.

DEITMER, J. W. & ELLIS, D. (1978). Changes in the intracellular sodium activity of sheep heart Purkinje fibres produced by calcium and other divalent cations. *Journal of Physiology* **277**, 437–453.

EISNER, D. A., ALLEN, D. G. & ORCHARD, C. H. (1985). Regulation of resting calcium concentration in cardiac muscle. In *Control and Manipulation of Calcium Movement*, ed. PARRATT, J. R., pp. 65–86. Raven Press, New York.

EISNER, D. A., LEDERER, W. J. & VAUGHAN-JONES, R. D. (1981). The dependence of sodium pumping and tension on intracellular sodium activity in voltage-clamped sheep Purkinje fibres. *Journal of Physiology* **317**, 163–187.

FABIATO, A. (1983). Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *American Journal of Physiology* **245**, C1–14.

FABIATO, A. (1985). Simulated calcium current can both cause calcium loading in and trigger calcium release from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *Journal of General Physiology* **85**, 291–320.

FEDIDA, D., NOBLE, D. & SPINDLER, A. J. (1988). Use-dependent reduction and facilitation of Ca^{2+} current in guinea-pig myocytes. *Journal of Physiology* **405**, 439–460.

FRAMPTON, J. E., ORCHARD, C. H. & BOYETT, M. R. (1990*a*). The relationship between diastolic and systolic intracellular [Ca^{2+}] in ventricular myocytes isolated from rat hearts. *Journal of Physiology* **425**, 53*P*.

FRAMPTON, J. E., ORCHARD, C. H. & BOYETT, M. R. (1990*b*). Caffeine-induced calcium release in myocytes isolated from the ventricles of rat hearts changes during inotropic interventions. *Journal of Physiology* **429**, 19*P*.

FRY, C. H., POWELL, T., TWIST, V. W. & WARD, J. P. T. (1984). Alterations to calcium exchange in digitonin-treated rat ventricular myocytes in response to changes in suspension pH. *Journal of Physiology* **346**, 78*P*.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. *Journal of Biological Chemistry* **260**, 3440–3450.

HANSFORD, R. G. & LAKATTA, E. G. (1987). Ryanodine releases calcium from sarcoplasmic reticulum in calcium tolerant rat cardiac myocytes. *Journal of Physiology* **390**, 453–467.

HARDING, D. P., KIRSCHENLOHR, H. L., METCALFE, J. C., MORRIS, P. G. & SMITH, G. A. (1989). The effect of stimulation frequency on end-diastolic $[Ca^{2+}]_i$ in isolated ferret heart. *Journal of Physiology* **417**, 51*P*.

HENRY, P. D. (1975). Positive staircase effect in the rat heart. *American Journal of Physiology* **228**, 360–364.

HOFFMAN, B. F. & KELLY, J. J. (1959). Effects of rate and rhythm on contraction of rat papillary muscle. *American Journal of Physiology* **197**, 1199–1204.

KIRBY, M. S., ORCHARD, C. & BOYETT, M. R. (1989). The control of calcium influx by cytoplasmic calcium in mammalian heart muscle. *Molecular and Cellular Biochemistry* **89**, 109–113.

KONISHI, M., HOLLINGWORTH, S. & BAYLOR, S. M. (1988). Myoplasmic binding of Fura-2 investigated by steady-state fluorescence and absorbance measurements. *Biophysical Journal* **54**, 1089–1104.

LADO, M. G., SHEU, S.-S. & FOZZARD, H. A. (1982). Changes in intracellular Ca^{2+} activity with stimulation in sheep cardiac Purkinje strands. *American Journal of Physiology* **243**, H133–137.

LANGER, G. A. (1968). Ion fluxes in cardiac excitation and contraction and their relation to myocardial contractility. *Physiological Reviews* **48**, 708–757.

LEE, H.-C. & CLUSIN, W. T. (1987). Cytosolic calcium staircase in cultured myocardial cells. *Circulation Research* **61**, 934–939.

LEE, H.-C., SMITH, N., MOHABIR, R. & CLUSIN, W. T. (1987). Cytosolic calcium transients from the beating mammalian heart. *Proceedings of the National Academy of Sciences of the USA* **84**, 7793–7797.

LEVI, A., PRICE, S., HALL, S. & KAUFMAN, A. (1990). Changes of coronary vessel resistance during enzymatic isolation of myocytes from guinea-pig hearts. *Journal of Physiology* **425**, 9*P*.

LI, Q., ALTSCHULD, R. & STOKES, B. T. (1987). Quantitation of intracellular free calcium in single adult cardiomyocytes by Fura-2 fluorescence microscopy: calibration of Fura-2 ratios. *Biochemical and Biophysical Research Communications* **147**, 120–126.

MARBAN, E. & WIER, W. G. (1985). Ryanodine as a tool to determine the contributions of calcium entry and calcium release to the calcium transient and contraction of cardiac Purkinje fibres. *Circulation Research* **56**, 133–138.

MEISSNER, G. (1986). Ryanodine activation and the inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. *Journal of Biological Chemistry* **261**, 6300–6306.

MITCHELL, M. R., POWELL, T., TERRAR, D. A. & TWIST, V. W. (1985). Influence of a change in stimulation rate on action potentials, currents and contractions in rat ventricular cells. *Journal of Physiology* **364**, 113–130.

MORAD, M. & TRAUTWEIN, W. (1968). The effect of the duration of the action potential on contraction in the mammalian heart muscle. *Pflügers Archiv* **299**, 66–82.

NOBLE, D. & POWELL, T. (1990). The attenuation and slowing of calcium signals in cardiac muscle by fluorescent indicators. *Journal of Physiology* **425**, 54*P*.

O'NEILL, S. C., DONOSO, P. & EISNER, D. A. (1990). The role of $[Ca^{2+}]_i$ and $[Ca^{2+}]$ sensitization in the caffeine contracture of rat myocytes: measurement of $[Ca^{2+}]_i$ and $[caffeine]_i$. *Journal of Physiology* **425**, 55–70.

O'NEILL, S. C. & EISNER, D. A. (1990). A mechanism for the effects of caffeine on Ca^{2+} release during diastole and systole in isolated rat ventricular myocytes. *Journal of Physiology* **430**, 519–536.

ORCHARD, C. H. & LAKATTA, E. G. (1985). Intracellular calcium transients and developed tension in rat heart muscle: A mechanism for the negative interval strength relationship. *Journal of General Physiology* **86**, 637–651.

ROE, M. W., LEMASTERS, J. J. & HERMAN, B. (1990). Assessment of Fura-2 for measurements of cytosolic free calcium. *Cell Calcium* **11**, 63–73.

SCANLON, M., WILLIAMS, D. A. & FAY, F. S. (1987). A Ca^{2+} insensitive form of Fura-2 associated with polymorphonuclear leukocytes. *Journal of Biological Chemistry* **262**, 6308–6312.

SCHOUTEN, V. J. A. & TER KEURS, H. E. D. J. (1986). The force–frequency relationship in rat myocardium: The influence of muscle dimensions. *Pflügers Archiv* **407**, 14–17.

SHEU, S.-S. & FOZZARD, H. A. (1982). Transmembrane Na^+ and Ca^{2+} electrochemical gradients in cardiac muscle and their relationship to force development. *Journal of General Physiology* **80**, 325–351.

SMITH, G. L., VALDEOLMILLOS, M., EISNER, D. A. & ALLEN, D. G. (1988). The effects of rapid application of caffeine on intracellular calcium concentration in ferret papillary muscles. *Journal of General Physiology* **92**, 351–368.

STEINBERG, S. F., BILEZIKIAN, J. P. & AL-AWQATI, Q. (1987). Fura-2 fluorescence is localized to mitochondria in endothelial cells. *American Journal of Physiology* **253**, C744–747.

SUTKO, J. L., ITO, K. & KENYON, J. L. (1985). Ryanodine: a modifier of sarcoplasmic reticulum calcium release. Biochemical and functional consequences of its actions on striated muscle. *Federation Proceedings* **44**, 2984–2988.

SUTKO, J. L. & WILLERSON, J. T. (1980). Ryanodine alteration of the contractile state of rat ventricular myocardium. Comparison with dog, cat and rabbit ventricular tissues. *Circulation Research* **46**, 332–343.

WEBER, A. M. & HERZ, R. (1968). The relationship between caffeine contracture of intact muscle and the effect of caffeine on isolated reticulum. *Journal of General Physiology* **52**, 750–759.

WENDT, I. R. & STEPHENSON, D. G. (1983). Effects of caffeine on Ca-activated force production in skinned cardiac and skeletal muscle fibres of the rat. *Pflügers Archiv* **398**, 210–216.

WIER, W. G., CANNELL, M. B., BERLIN, J. R., MARBAN, E. & LEDERER, W. J. (1987). Cellular and subcellular heterogeneity of $[Ca^{2+}]_i$ in single heart cells revealed by Fura-2. *Science* **235**, 325–328.

WILLIAMS, D. A., FOGARTY, K. E., TSIEN, R. Y. & FAY, F. S. (1985). Calcium gradients in single smooth muscle cells revealed by digital imaging microscopy using Fura-2. *Nature* **318**, 358–361.